Faculdade de Ciências do Mar e do Ambiente

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Nutritional modulation of innate immune parameters in the epidermal mucus of Senegalese sole (*Solea senegalensis*)

Influência da dieta nos parâmetros da resposta imunitária inata do muco de linguado (*Solea senegalensis*)

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This thesis is dedicated to my parents without whom none of this would have been possible.

Vera Rodrigues

Abstract

Fish are in constant interaction with their habitat and potential pathogens. Fish epidermis acts as the first line of defense, since mucus secreted by mucous cells contains innate immune parameters, such as enzymes and antimicrobial proteins. Knowledge about the mechanisms of immune defense at the skin and mucus level in fish is still scarce.

This study was undertaken to evaluate the role of nutritional factors as tools to enhance the immune parameters in the mucus of Senegalese sole (*Solea senegalensis*). Homogenous groups of Senegalese sole (initial body weight: 92.8 $\pm$ 1.52g) were fed: a control diet (CTRL); the CTRL diet supplemented with vitamin C (1000 mg/kg) (VIT C); and a diet identical to CTRL but in which 70% of the dietary fat was originated from coconut oil (COC), identified as a potential immunostimulant. After a period of 4 and 6 weeks of experimental feeding, fish mucus was sampled for quantification of lysozyme, alkaline phosphatase, proteases, lipid antioxidation (TBARS) and antimicrobial activity. Growth performance and lysozyme activity was not affected by dietary treatments, while the proteolytic activity and lipid oxidation was affected positively by both dietary treatments. Alkaline phosphatase activity was only affected by diets rich in coconut oil. Epidermal mucus of sole showed antibacterial activity against a series of marine pathogen bacteria. Highest inhibitory action was associated to mucus extracts derived from coconut oil fed fish.

The modulation of selected mucus immune parameters through dietary factors is possible in Senegalese sole. A four weeks period of feeding seems enough to induce such changes. Further research is needed to determine the processes associated to such modulation and evaluate to what extent these beneficial changes contribute to an enhanced immune response of sole.

Resumo

Os peixes estão em constante interacção com o seu habitat, e potenciais agentes patogénicos. A epiderme dos peixes actua como primeira linha de defesa, uma vez que o muco secretado pelas células secretoras de muco contém factores da resposta imunitária inata, tais como enzimas e proteínas antimicrobianas. O conhecimento sobre os mecanismos da defesa imunológica ao nível da pele e do muco dos peixes é reduzido.

Este estudo foi realizado com o objectivo de avaliar o papel dos factores nutricionais como ferramentas para estimular os parâmetros imunológicos em muco de linguado (*Solea senegalensis*). Grupos homogéneos de linguado (peso inicial: 92.8 $\pm$ 1.52g) foram alimentados: com uma dieta controlo (CTRL); a dieta CTRL suplementada com vitamina C (1000 mg/kg) (VIT C); e uma dieta idêntica ao CTRL mas na qual 70% da gordura provinha de óleo de coco (COC), identificada como tendo um efeito imunoestimulante. Após um período de 4 e 6 semanas de alimentação experimental, foram recolhidas amostras de muco epidérmico e quantificadas a actividade da lisozima, fosfatase alcalina, proteases, oxidação lipídica (TBARS) e a actividade antibacteriana. O crescimento e a actividade da lisozima não foram afectados pelos tratamentos alimentares, enquanto que as proteases e a oxidação lipídica foram afectadas positivamente pelos dois tratamentos. No caso da actividade da fosfatase alcalina, verifica-se apenas um aumento da sua actividade com a dieta suplementada em óleo de coco. O muco epidermal do linguado mostra actividade antibacteriana contra uma série de bactérias marinhas patogénicas, e o maior grau de inibição está associada ao extrato de muco derivado de peixes alimentados com a dieta com óleo de coco.

A modulação por via nutricional de alguns parâmetros imunitários no mucus do linguado é possível. Um período de quatro semanas parece ser suficiente para induzir tais mudanças. São necessários estudos complementares para determinar os processos associados a esta modulação e avaliar em que medida estas mudanças serão benéficas como contributos para o reforço da resistência do linguado a patologias.

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Although the thesis has in its cover only the author's name, none of this work would have been even possible to accomplish without the hefty support of some people, that I fear I may unintentionally forget here. If that is the case, please accept my sincere apologies. I sincerely hope that anyone who in anyway contributed and helped me get this far will share with me this particular moment of happiness and satisfaction which I am now enjoying.

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1. Introduction

Aquaculture is the farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants under controlled conditions. Aquaculture, probably the fastest growing food-producing sector, now accounts for almost 50 percent of the world's food fish supply and is perceived as having the greatest potential to meet the growing demand for aquatic food. Given the projected population growth over the next two decades, it is estimated that at least an additional 40 million tons of aquatic food will be required by 2030 to maintain the current per capita consumption (FAO, 2006).

Aquaculture has evolved into an industrial activity. As a consequence higher productivity has been closely associated to an intensification of production conditions (e.g. higher rearing densities). As a result, production losses due to diseases have also increased, given the enhanced exposure of fish to pathogens, such as bacteria, parasites, or virus, throughout the production cycle (Laidler et al., 1999).

Infectious diseases are the major cause of economic losses in commercial aquaculture. Current methods for treating diseases in fish include a limited number of government-approved antibiotics and chemotherapeutics that have in most cases limited effectiveness. As such, the aquaculture industry has begun to focus on prevention of disease rather than treatment. One undisputed concept behind such approach is the fact that to avoid disease epidemics in aquaculture, fish need to be reared in good environmental conditions and priority should be given to the optimization of their welfare status (Mozumder, 2005).

Fish welfare must be assured, by guaranteeing that the fish live as well as possible and express their natural behavior as much as possible, free from negative experiences (stressors). Given that fish are in direct contact with their environment through the large surface of their gills and skin, water quality (dissolved oxygen, CO_2, ammonium and pH) and the presence of contaminants (organics and inorganic pollutants) are some of the most critical aspects of the environment for fish welfare (Mellor & Stafford, 2001). Furthermore, under captivity, fish are also subjected to a

great number of other potential stress factors, such as husbandry and feeding practices.

1.1. The farming of Senegalese sole (*Solea senegalensis*)

Senegalese sole (*Solea senegalensis* Kaup, 1858) has longtime been considered a promising candidate species for marine aquaculture in the Mediterranean and South Atlantic costal areas (Figure 1). Senegalese sole is commonly raised in extensive polyculture (in earth ponds) in the South of Portugal and Spain, where it can achieve higher growth rates than European seabass (*Dicentrarchus labrax*), being second only to gilthead seabream (*Sparus aurata*). Sole's high commercial value has motivated farmers to initiate its intensive farming. This interest has gain momentum in Southern Europe over recent years, as a result of the rapid increase in the production of European seabass and gilthead seabream leading to market saturation and a decline in profitability for these traditional species (Morais et al., 2006).

Figure 1. The Senegalese sole (Solea senegalensis).

Significant progress on sole farming has been obtained on aspects related to spawning-control under captivity, the development of feeding protocols, in particular during early life stages, and the estimation of nutritional requirements of larvae and juveniles. Over the last years, research effort has been put into optimizing larval rearing techniques for this species, since this was considered as the bottleneck, hampering the development of commercial scale culture. At present, the main problem associated to the large scale cultivation of sole it is the high sensitivity of the post-larvae and juveniles to stressful situations commonly found in aquaculture activities, such as grading,

weighing or initial feeding with artificial diets. These stressful conditions cause not only sub-optimal growth, but also a high susceptibility to opportunistic pathogens, mainly *Flexibacter*, which may lead to mass mortalities. Being a new species to aquaculture, knowledge on the mechanisms associated to disease resistance and stress response in sole are extremely scarce.

1.2. The immune response in fish

Fish are a diverse group of animals specialized for life in the aquatic environment. Their connection with their habitat is very close and so they are always in contact with bacteria, viruses and fungi that can appear in large concentrations, be pathogenic and cause damage to these organisms. However, under normal conditions the fish can maintain its well state, defending itself against potential invaders, because of a complex system of defense (Mozumder, 2005; Arellano et al., 2004).

The immune system is divided into innate (non-specific) and acquired (specific) (Figure 2). The first is the only weapon of defense of invertebrates and a key defense mechanism of fish. It is of vital importance in resistance to diseases, especially given that the innate response generally precedes the adaptive response, activates and determines the nature of the adaptive response and co-operates in the maintenance of homeostasis (Magnadóttir, 2006). The specific immune mechanisms in fish are slow and limited by temperature constrains on their metabolism. So, fish are likely to rely highly on their innate immune mechanisms for protection against invading pathogens (Bly & Clem, 1991; Ellis, 2001; Ingram, 1980).

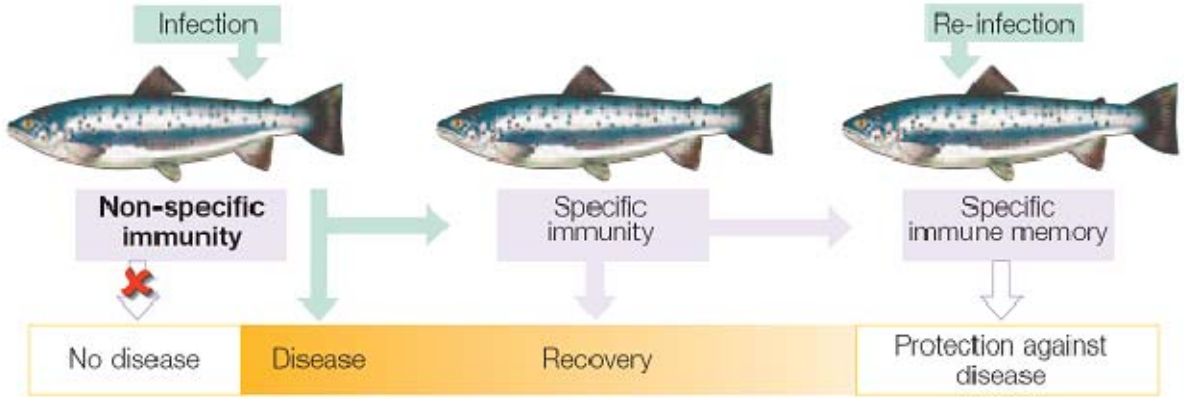


Figure 2. Non-specific and specific immune responses and protection of fish against infection. Source: DSM Nutritional Products brochure "The effect of vitamin C on fish health".

The innate system is divided in physical barriers (scales, mucus surfaces of skin, gills, gut and the epidermis which act as the first barriers against infection), cellular (phagocytic cells and the non-specific cytotoxic cells) and humoral components (growth inhibitors, various lytic enzymes and components of the complement pathways, agglutinins and precipitins, natural antibodies, cytokines, chemokines and antibacterial peptides) (Magnadóttir, 2006; Subramanian et al., 2007).

1.3. Mucus as a natural immune barrier in fish

The epidermis is a metabolically very active border tissue containing mucous, sensory, chloride and club cells, and is covered by a mucous layer that forms an additional external barrier containing enzymes (Arellano et al., 2004). This mechanical barrier impedes the entry of the majority of microorganisms into the body and the lining of the alimentary, respiratory, and urogenital tracts as well as total epidermis. Together, the skin and mucus represent the first point of contact between host and potential pathogen (Mozumder, 2005).

In the skin of Senegalese sole, four morphologically distinct layers were identified: cuticle, epidermis, dermis and hypodermis (Figure 3). The epidermis is composed of stratified epithelium, containing three cellular layers: the outermost or mucosa layer, the middle or fusiform layer and the stratum germinativum or the basal layer. In the mucosa, two mucous cell types are

differentiated: type-A cells containing several round vesicles of different electron density and type-B cells containing mucosomes of uniform electron density (Arellano, 2004).

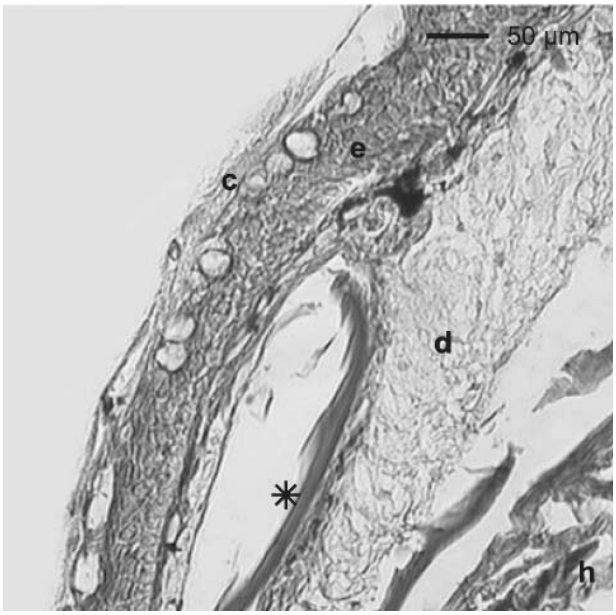


Figure 3. Section of Senegalese sole skin.
*(C, cuticle; E, epidermis; D, dermis; H, hypodermis; * fish scale (haematoxylin, H/VOF)*
Source: Arellano, 2004.

The epidermal mucus is produced primarily by epidermal goblet or mucus cells and is composed mainly of water and gel-forming macromolecules, including mucins and other glycoproteins (Shephard, 1993), and presents a diversity of functions in fish, including: protection against and resistance to disease, respiration, ionic and osmotic regulation, excretion, communication and feeding (Ingram, 1980; Shephard, 1994; Arellano et al., 2004). In recent years it has been demonstrated that mucus plays an important role in the prevention of colonization by parasites, bacteria and fungi (Mozunder, 2005).

The information available on the antimicrobial function of epidermal mucus is restricted to a few fish species. For example, Austin & McIntosh (1988) demonstrated the existence of antimicrobial property in the epidermal mucus of rainbow trout (*Oncorhynchus mykiss*); in ayu (*Plecoglossus altivelis*) and turbot (*Scophthalmus maximus*) the removal of epidermal mucus after challenging

them with *Listonella anguillarum* resulted in increased mortality (Kanno et al., 1989, Fouz et al., 1990). Lemaître et al. (1996) observed in their work in carp (*Cyprinus carpio*) that loss of epidermal mucus increased susceptibility to bacterial infection. Antimicrobial activity of epidermal mucus extracts against a broad range of microbial pathogens was observed by Hellio et al. (2002). All these experiments support the hypothesis that the epidermal mucus plays a protective function against microbial infection in fish (Subramanian et al., 2008).

The antimicrobial function of epidermal mucus appears to result from its mechanical and biochemical properties (Pickering, 1974). As an element of the innate immune mechanism, mucus has a dual function: presents a continuous production, thereby preventing the adherence of pathogens, and also acts as a repository of several innate immune factors: lysozymes, immunoglobulins, complement proteins, lectins, C-reactive proteins, proteolytic enzymes and various other antibacterial proteins and peptides (Subramanian et al., 2008; Palaksha et al., 2008; Fast et al., 2002; Ross et al., 2000; Cole et al., 1997). Altered expression of these different non-specific factors was associated with variations in resistance between species (Fast et al., 2002).

Little is known about the role of enzymes in the epidermal mucus in the innate immune system of some species of fish (Subramanian et al., 2008), but there are many indications that in the mucus layer are present many antimicrobial enzymes and proteins, which are involved in innate immunity of fish (Mozunder, 2005). Innate immune parameters such as lysosyme, proteases, and cathepsins have been used as indicators of disease resistance of fish (Magnadóttir, 2006).

Lysosyme is the best know bacteriolytic in non-specific defense in animals and fish, aiding in the prevention of bacterial infection (Aranishi, 1999). It is present in mucus, lymphoid tissue, plasma and other body fluids in most species of fish (Magnadóttir, 2006). Also referred as N-acetylmuramide glycanohydrolase or muramidase, it functions by hydrolyzing the ß-(1-4) linkage between N-acetylmuramic acid and 3-acetyl amino-2-deoxy-D-glucose residues of mucopolysaccharide found in bacterial cell walls (Magnadóttir, 2006, Subramanian et al., 2007). It acts directly on the walls of Gram-positive bacterial cells, causing lysis of its outermost

peptidoglycan layer, and in Gram-negative bacterial cells acting subsequently to the degradation of the outer membrane, by complementing other enzymes that expose the peptidoglycan layer (Yano, 1996). This enzyme has been reported in the mucus of many species such as Japanese flounder (*Paralichthys olivaceus*), coho salmon (*O. kisutch*), koi carp (*Cyprinus carpio*), haddock (*Melanogrammus aeglefinus*), Atlantic cod (*Gadus morhua*), hagfish (*Myxine glutinosa*), Arctic char (*Salvelinus alpinus*), brook trout (*S. fontinalis*), striped bass (*Morone saxatilis*) and others (Hikima et al., 1997; Schrock et al., 2001; Subramanian et al., 2007).

Alkaline phosphatase (AP), another enzyme present in the mucus, has been established as a potential stress indicator in the epidermal mucus of Atlantic salmon (*S. salar*). It is also thought to act in a protective role in the initial stage of wound healing in carp and as an antibacterial agent because of its hydrolytic activity (Ross et al., 2000; Fast et al, 2002; Subramanian et al., 2007).

Proteases have a significant role in the innate immune mechanisms (Ingram, 1980). They can cleave bacterial proteins thus directly damaging the pathogen. They also function indirectly by activating and enhancing the production of various immunological components such as complement, immunoglobulin and antimicrobial peptides. Proteases are classified into serine, cysteine, aspartic and metalloproteases based on the chemical nature of the groups responsible for catalysis. Fish mucus has been found to have serine proteases (trypsin); cysteine proteases (cathepsins B and L); aspartic proteases (cathepsin D) and metalloproteases (Subramanian et al. 2007). Several proteases that have been observed in fish have been correlated with one or more of the above mentioned activities. For instance, a cathepsin D found in the mucus of catfish (*Parasilurus asotus*), has been shown to activate the production of the antimicrobial peptide parasin I (Cho et al., 2002). Moreover, cathepsins B and L showed bacteriolytic activity against the fish pathogens *Edwardsiella tarda*, *Flavobacterium columnare* and *L. anguillarum* (Aranishi, 1999).

Oxidative stress is an inevitable result of life in an oxygen-rich environment. For aerobic organisms, oxygen is paradoxically both vital and dangerous. The so-called “oxygen paradox” derives from the chemical nature of oxygen, which in its atomic form (O) is a free radical and in its molecular form (O_2) is a bi-radical (Davies, 2000). Aerobic tissues continuously generate superoxide radicals and hydrogen peroxide (H_2O_2) as by-products of oxidative metabolism (Passi et al., 2004). Oxidative stress occurs when reactive oxygen species (ROS) generation exceeds its removal. The deleterious effects include oxidation of proteins, DNA, steroid components, as well as peroxidation of unsatured lipids in cell membranes (Martínez-Álvarez et al., 2005). In particular, ROS are able to start the lipid peroxidation process, which is potentially dangerous in fish, since they contain a high percentage of polyunsatured fatty acids. Lipid peroxidation is involved in a variety of disorders and pathological conditions (Nakamura et al., 1998). Intrinsic factors, such as age, phylogenetic position, nutritional status and environmental factors such as dissolved oxygen, temperature, toxins present in the water, pathologies, parasites, can either enhance or decrease antioxidant defences in fish (Martínez-Álvarez et al., 2005).

1.4. The potential of immunostimulants in fish

The goals of the aquaculture industry are to optimize growth and to produce high-quality fish. As in all farming, the outbreak of diseases in fish farming can be a major concern. The high susceptibility of fish to stress and the rapid spread of diseases in water have forced fish farmers to concentrate their efforts on maintaining fish in good health in order to achieve sustainable economic performances.

Growing healthy fish requires them to be able to develop strong defense mechanisms against pathogen invasion. Intensively raised fish may be exposed to stressful situations which often result in a depressed immune status. Good management practices reduce stress and therefore help to maintain healthy animals. However, since not all stressing situations can be avoided, fish with enhanced defense mechanisms will be better prepared to combat the negative effects of stress. The nutritional quality of the feed is a major factor in sustaining healthy fish. It has been shown that the immune system can be enhanced by the use of immunostimulants such as antioxidant

vitamins, carotenoids and other feed additives (Vadstein, 1997). The combination of good management, vaccination and nutritional prophylaxis will insure higher survival rates and improve growth in intensive farming systems (Figure 4).

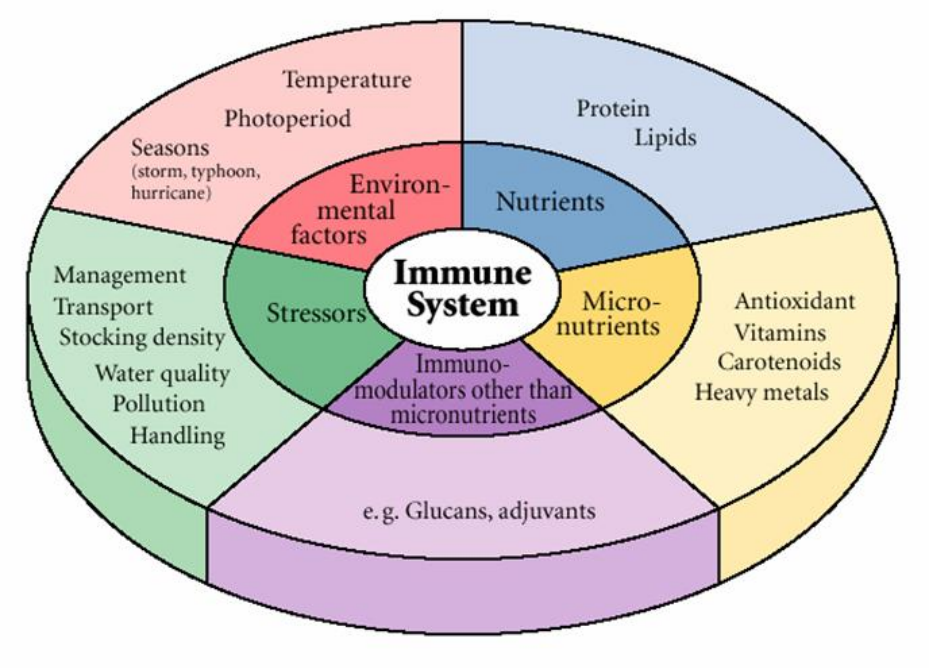


Figure 4. Schematic view of factors influencing the immune response in fish.
Source: DSM Nutritional Products brochure "The effect of vitamin C on fish health".

Bricknell & Dalmo (2005) define immunostimulant as "a natural occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens". Sakai (1990) divided the immunostimulants into several groups depending on their sources: bacterial, algae-derived, animal-derived, nutritional factors as immunostimulants, and hormones/cytokines. These compounds may directly initiate activation of the innate defense mechanisms acting on receptors and triggering intracellular gene activation that may result in production of antimicrobial molecules (Bricknell & Dalmo, 2005).

Nutritional status is considered one of the important factors that determine the ability of fish to resist diseases. Nutritional modulation of resistance to infectious diseases, based upon the information on fish and terrestrial animals, is divided into four major groups. In the first category,

one must consider a proper balance of macro- and micronutrients, including amino acids, polyunsaturated fatty acids (PUFA), vitamins and trace elements, which are essential for the development of immune system starting at the larval stage. Deficiencies in these nutrients may impact several development events including the proper development of lymphoid organs. Marginal deficiencies may negatively affect the immune system at later stages of life. Severe deficiencies will increase susceptibility to disease and may result in the death of the animal. The second point is to consider that adequate nutrition is essential for cells of the immune system to divide and synthesize effector molecules. The diet supplies the immune system with the amino acids, PUFA, enzyme co-factors and energy necessary to support lymphocyte proliferation and the synthesis of effector (e.g. immunoglobulins, lysozyme and complement) and communication molecules (e.g. cytokines and eicosanoids). The quantitative need for nutrients to maintain a normal immune function is relatively small compared to the requirements for growth and reproduction. In the third category, it is important to consider that some nutrients provide essential substrates for the proliferation of pathogens (e.g. iron) and their presence at low concentrations in body fluids may limit the growth of pathogens within the fish. The fourth mechanism may include the indirect regulatory effects of diets on the immune system that are mediated through the endocrine system (Lall, 2000).

1.5. Objectives of the study

This study was undertaken to evaluate the role of nutritional factors as tools to enhance selected immune parameters in the mucus of Senegalese sole.

The nutritional variables chosen were:

- ***Vitamin C level***

Vitamin C (ascorbic acid, AA) is an essential vitamin for normal growth, physiological functions and plays an important role in immunity in animals including fish (Lin & Shiau, 2005; Ren et al, 2007). It is the most important water-soluble antioxidant and is a cofactor in many hidroxylating reactions (Kumari & Sahoo, 2005). The influence of AA on the immune response may be related to its antioxidant activity as a free radical scavenger, protecting cells from auto-oxidation and maintaining their integrity for an optimal functioning of the immune system. Since ascorbic acid is involved in several enzymatic processes, it is difficult to determine the exact mechanism of action behind the stimulation of the immune response (Kumari & Sahoo, 2005). Dietary supplemental levels of vitamin C have been shown to enhance immune status and disease resistance, in a wide variety of species, such as channel catfish (*Ictalurus punctatus*), Indian major carp (*Labeo rohita*), rainbow trout, Atlantic salmon, Japanese seabass (*Lateolabrax japonicus*) and large yellow croaker (*Pseudosciaena crocea*) (Sahoo & Mukherjee, 2003; Ai et al., 2004; Lin & Shiau, 2005; Kumari & Sahoo, 2005; Ai et al., 2006). A number of studies have also shown positive effects of vitamin C on several non-specific immunological parameters, such as lysosyme, respiratory burst, and resistance to stress and diseases (Verlhac et al., 1996; Sahoo & Mukherjee, 2003; Ai et al. 2004; Lin & Shiau, 2005; Kumari & Sahoo, 2005; Ai et al; 2006).

- ***Coconut oil***

Recent studies show that fatty acids are important effectors in the immune functions (Puertullano et al., 2004). Dietary lipids may modulate a great number of immune parameters, such as lymphocyte proliferation, cytokine synthesis, natural killer cell activity, phagocytes and others

(Pablo & Cienfueros, 2000). Coconut oil is constituted by a unique blend of several fatty acids (50% lauric acid and 7% capric acid), which show potent antiviral, antibacterial and antiprotozoal functions. In general, it is reported that fatty acids and monoglycerides produce their killing/inactivating effect by lysing the plasma membrane of lipid bilayer. The antiviral action attributed to monolaurin is that of solubilizing the lipids and phospholipids in the envelope of the virus, causing its disintegration. However, there is evidence from recent studies that one antimicrobial effect in bacteria is related to monolaurin interference with signal transduction (Projan et al., 1994), and another antimicrobial effect in viruses is due to lauric acid interference with virus assembly and viral maturation (Hornung et al., 1994; Isaacs et al., 1992). To our knowledge the potential of coconut oil as an immune effector has never been studied in fish.

2. Materials and Methods

2.1. Experimental diets

Three experimental diets were formulated to fulfill the nutritional requirements of Senegalese sole. A control diet (CTRL diet) was a practical formulation currently used for sole farming, with 150 mg/kg of vitamin C. The control formula was supplemented with vitamin C at 1000 mg/kg (VIT C diet) and a third formula, identical to control but in which 70% of the original dietary fat source (fish oil) was replaced by coconut oil (COC diet). The formulation in terms of ingredients and the proximate composition of the experimental diets are presented in Table I.

Table I. Formulation and composition of the experimental diets.

Ingredients (%)	CTRL	COC	VIT C
Fishmeal LT	35.00	35.00	35.00
Fish protein concentrate (CPSP G)	12.50	12.50	12.50
Soybean meal 48	16.00	16.00	16.00
Corn gluten	8.00	8.00	8.00
Wheat meal	21.00	21.00	20.73
Coconut oil	0.00	5.00	0.00
Fish oil	7.00	2.00	7.00
Choline chloride 50%	0.10	0.10	0.10
Vitamin C (Lutavit C35)	0.03	0.03	0.30
Vitamin E (Lutavit E50)	0.05	0.05	0.05
MIN & Vit Mixture	0.25	0.25	0.25
Betaine	0.07	0.07	0.07
Proximate composition			
Dry matter (DM) (%)	92.47	91.25	92.33
Crude protein (%DM)	46.21	46.21	46.19
Crude fat (%DM)	11.93	11.93	11.93
Gross Energy (kJ/g DM)	20.79	20.79	20.75
Vitamin C (ascorbic acid) (mg/kg DM)	141.00	144.00	1093.00

Ingredients were finely ground, mixed in an horizontal helix ribbon mixer (model Mano, 100 L capacity, CPM, San Francisco, USA) for 15 min and pelleted dry using a steamless pelleting machine (model 3000, CPM, San Francisco, USA) fitted with a die of 3.0 mm in diameter. Pellets were dried

at room temperature and subsequently stored at 4ºC. Diets were manufactured at the Zootechnical Departement of the University of Trás-os-Montes e Alto Douro.

2.2. Experimental animals and rearing conditions

Three homogeneous groups of 20 Senegalese sole (initial body weight: 92.8 ± 1.52 g) were maintained at the Experimental Station of Ramalhete (University of Algarve), in 3 rectangular PVC tanks (31 x 55 x 94 cm); volume: 160 L; water-flow rate: 3.6 $L \cdot min^{-1}$), supplied with recirculated seawater (constant temperature: 19.2 ± 1.6ºC; salinity: 36.2 ± 1‰; dissolved oxygen: 94.1 ± 17.6 $mg \cdot L^{-1}$). A photoperiod of 13/11 fluorescent light/dark cycle was adopted, with a light intensity of 90 Lux. Fish were fed with one of the three experimental diets by means of automated feeders for 12 hours (10.00 till 22.00) during 6 weeks.

2.3. Mucus collection

Fish used in this study were individually measured and weighed at the beginning and end of the experiment. After 4 and 6 weeks of experimental feeding, the epidermal mucus was collected in individual fish according to the method initially described by Ross et al. (2000) and slightly modified by Subramanian et al. (2007). The fish were starved for 24 hours prior to sampling. The mucus samples were obtained from 18 fish per tank and aggregated in pools of 3 fish. The fish were anaesthetized with a sub-lethal dose (150 $mg \cdot L^{-1}$) of 2-phenoxy-ethanol (Sigma-Aldrich). Each individual fish was then placed in a plastic bag with zip (Figure 5) containing 5 ml of 100 mM NaCl. The bags were then gently shaken by hand during 1-2 min and afterwards fish were carefully placed in a recovery tank.

Figure 5. Mucus collection in Senegalese sole.

The mucus sample in the bag was transferred to a 50 ml sterile centrifuge tube and placed in ice. The mucus remaining in the bag was rinsed with additional 5 ml of NaCl and pooled in the same tube (Figure 6).

Figure 6. Transfer of mucus from the collecting bag to a 50 ml sterile centrifuge tube.

The mucus samples were immediately transported to the laboratory, where they were centrifuged at 2000 x *g* for 15 min at 4ºC. The pellet was eliminated and the supernatant was homogenized with a sonicator for 20 seconds, aliquoted in 2 ml eppendorf tubes and stored at -80ºC until subsequent assay of total protein, lysozyme activity and lipid oxidation (TBARS). Prior to these assays, the thawed samples were further centrifuged at 8850 x *g* for 2 min at 4ºC. Only this last supernatant was used for assays. For alkaline phosphatase (AP) and protease activity the initial supernatant was aliquoted in 1 ml samples, freeze dried and stored at -80 ºC until further use. Prior to the assay, samples were reconstituted to 1 ml with the respective enzyme assay buffers, centrifuged at 8850 x *g* for 2 min at 4ºC. Only this last supernatant was used for assays (Figure 7).

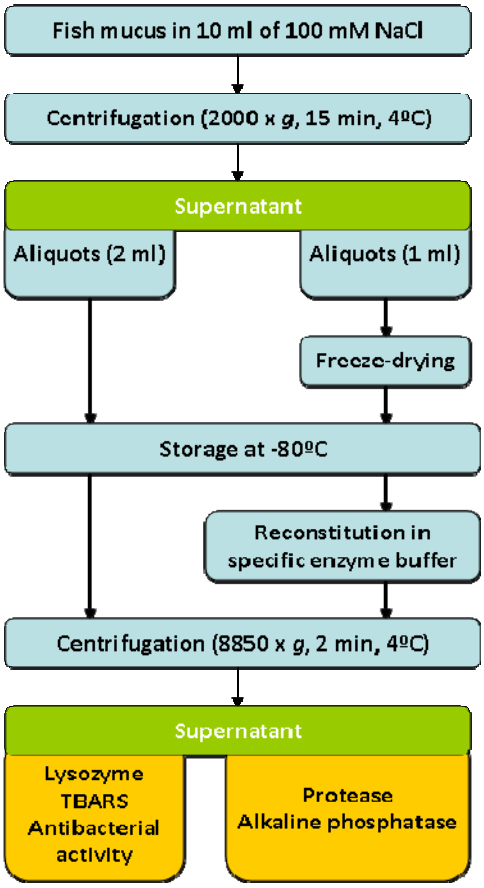


Figure 7. Flow chart of sample preparation and analytics.

2.4. Analytical methods

2.4.1. Protein

Protein concentration of mucus samples was determined by spectrophometry measurement of protein-dye binding according to the Bradford method (Bradford, 1976). The total protein concentration determined was then used to calculate the specific activity for the various assays. The absorbance for various assays was read using a microplate reader BioRad Benchmark.

2.4.2. Lysozyme

Lysozyme activity was determined using a turbidimetric assay adapted from Ross et al. (2000), with some modifications. Twenty-one mg of lyophilized *Micrococcus lysodeikticus* bacterial cells (Sigma) were suspended in 100 ml of 0.05 M Na-phosphate buffer (50 ml sodium phosphate dibasic dehydrate and 200 ml potassium phosphate monobasic), adjusted to pH 6.2 with HCl. This solution was made fresh everyday. The *Micrococcus lysodeikticus* suspension was incubated in a water bath at 30 ºC under gentle stirring for not less than 30 minutes. 50 µl of both buffer (for blank) and sample were pipetted into a 96-well microplate in triplicate. 150 µl of the bacterial solution was rapidly added using a multi-dispenser pipette and absorbance was read after 5 and 20 min at 450 nm. One unit of lysozyme activity was defined as the amount of enzyme that catalyzed a decrease in absorbance of 0.001 min^{-1}. Activity units were expressed per mg of protein (specific activity).

2.4.3. Alkaline phosphatase

Alkaline phosphatase (AP) activity was determined according to Ross et al. (2000). Fifty µl of mucus reconstituted in 100 mM ammonium bicarbonate, 1 mM magnesium chloride hexahydrate, pH 7.8 buffer were incubated in a 96-well plate Thermo Shaker (Grant) at 30 ºC for 15 min. 150 µl of 4 mM p-nitrophenol phosphate substrate was rapidly added using a multi-dispenser pipette. Absorbance was read at 405 nm after 20 and 25 minutes of reaction. One unit of activity was defined as the amount of enzyme required to release 1 nmol of p-nitrophenol in 1 min. The extinction coefficient of p-nitrophenol in the microplate wells was experimentally determined using a standard curve with concentrations ranging 0-50 µM. Activity units were expressed per mg of protein (specific activity).

2.4.4. Proteases

Protease activity was determined using the azocasein hydrolysis assay according to Firth et al., (2000). Azocasein hydrolysis was assayed by incubating 50 µl of the mucus sample re-suspended in 100 mM ammonium bicarbonate, pH 7.8, with 50 µl azocasein substrate 0.25% (w/v) in the same buffer for 19 h at 30 °C. The reaction was stopped by adding 50 µl of 20% (w/v) trichloroacetic acid followed by a 5 min centrifugation at 15400 × g. Equal volumes (100 µl) of the resultant supernatant and 0.5 M NaOH were added to a 96-well plate and the absorbance measured at 405 nm. One unit of activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 min^{-1}. Activity units were expressed per mg of protein (specific activity).

2.4.5. Lipid oxidation (TBARS)

Oxidation of lipids was determined in mucus by measuring the thiobarbituric acid reactive substances (TBARS) according to the method adapted from Burk et al. (1980). TBARS was assayed by incubating 750 µl of the mucus samples with 1500 µl of trichloroacetic acid 20% and 50 µl 1% butylated hydroxytoluene (BHT) in methanol. A standard curve with concentrations ranging from 0 to 5000 nmol of MDA solution (1,1,3,3-tetraethoxypropane) was also assayed. Both samples and standards were incubated with 2950 µl of 2-thiobarbituric acid 50 mM in a 90 ºC water bath for 20 min. After cooling and centrifugation at 2000 x *g* for 20 min at 4 ºC, supernatants (200 µl) were transferred to a 96-well plate and absorbance read at 540 nm. TBARS concentration was calculated by correspondence to the standard curve, and expressed per mg of protein.

2.4.6. Antibacterial assay

The antibacterial assays were done by disc-assay method (Chabbert, 1963). Mucus extracts (100 ul) were impregnated in sterile filter paper discs (6 mm in diameter, Schleicher and Schuell, Inc.). Discs were allowed to evaporate and then placed on Zobell's agar test plates (9 cm diameter) inoculated with 18 hours culture of the test pathogens (10^7 bacteria /ml) in 15‰ NaCl TSA broth. The plates were then incubated for 48 h at 22.8 ºC before reading the inhibition zone diameter. The antibacterial tests were performed in triplicate against five Gram-negative bacteria *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Yersinia ruckeri*, *Vibrio alginolyticus* and *Listonella anguillarum*. The test bacterial pathogen cultures were obtained from stock cultures maintained at the Microbiology Laboratory of the Unity of Upgrading of Fishery and Aquaculture Products, from the National Institute of Biological Resources (Table II).

Table II. Characteristics of the bacterial strains tested.

Name	Origin	Characteristics
Aeromonas hydrophila	INRB – IPIMAR	Isolated from trout
Aeromonas salmonicida	IFREMER	
Listonella anguillarum	IFREMER	Serotype I
Vibrio alginolyticus	INRB – IPIMAR	ATCC 17749
Yersinia ruckeri	Institute Pasteur Collection	CIP: 8280T

The zone of inhibition of bacteria around the disc was recorded using Vernier calipers (Figure 8). The assay was scored positive (+) if it was < 2 mm, doubly positive (++) if the zone was ≥ 2 mm, triple positive (+++) if the zone of inhibition was ≥7 mm, and negative (-) if there was no inhibition of microbial growth (Thompson et al., 1985). The bacterial assay results were compared with those obtained when challenging the bacterial pathogens with two reference antibiotics (i.e. discs containing chloramphenicol 30 mg, oxytetracycline 30 UI from Sanofi Diagnostics Pasteur discs).

Figure 8. Antibacterial potential of sole epidermal mucus assayed by the disc-diffusion method.

2.4.7. Statistical analysis

Data are presented as means ± standard deviation. Data were subjected to a two-way analysis of variance, considering dietary treatment and duration of feeding as variables, and when appropriate, means were compared by the Newman-Keuls test. Parameters expressed as percentages were subjected to arcsin square root transformation. Statistical significance was tested at 0.05 probability level. All statistical tests were performed using the STAGRAPHICS Centurion XV, version 15.1.02 software.

3. Results

3.1. Growth performance

Data on growth performance of sole fed the different experimental diets during 48 days are reported in Table III. Specific growth rate (SGR) values ranged from 0.38 to 0.63 %/day and neither weight gain nor growth rates were significantly affected (P>0.05) by vitamin C supplementation or coconut oil inclusion. However it is worth mentioning a slight trend to a growth enhancement observed in fish fed the coconut oil diet (COC). Still, the high weight dispersion that generally characterizes sole rearing makes it difficult to attain statistical significance. Similarly to what is observed in terms of weight, the condition factor (K) of fish was not affected by the various dietary treatments. Moreover, K values were not altered between the beginning and the end of the trial.

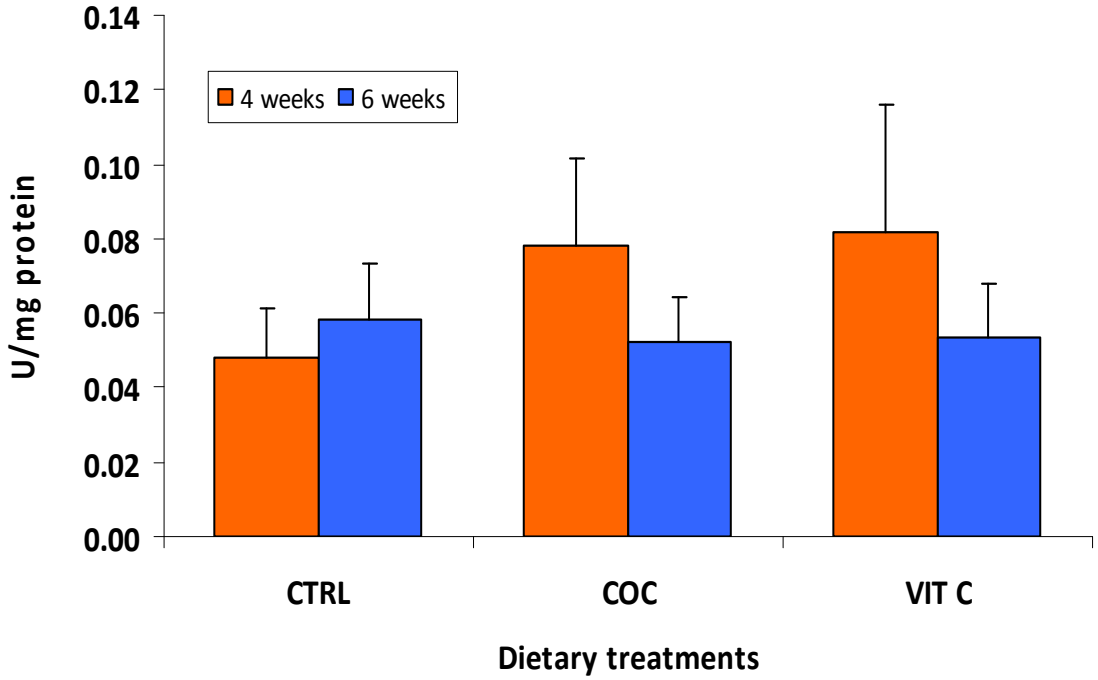
Table III. Growth performance of sole fed the various dietary treatments over 6 weeks.

	Dietary treatments		
	CTRL	COC	VIT C
Initial body weight, g	92.5 ± 21.9	94.5 ± 19.1	91.5 ± 13.8
Final body weight, g	113.3 ± 26.0	130.0 ± 28.6	116.4 ± 16.6
Weight gain, %IBW/d	0.42 ± 0.25	0.76 ± 0.30	0.61 ± 0.34
SGR, %/d	0.38 ± 0.20	0.63 ± 0.22	0.52 ± 0.27
Initial total length, cm	17.5 ± 1.5	17.7 ± 1.5	17.9 ± 0.9
Final total length, cm	18.9 ± 1.5	19.6 ± 1.4	19.3 ± 0.9
K	1.66 ± 0.19	1.71 ± 0.24	1.61 ± 0.16

Values are means ± sd.
Specific growth rate: SGR = 100 x (Ln final body weight - Ln initial body weight) / 48 days.
Condition factor: K = (100 x total weigh) / total length^3

3.2. Immune parameters

The presence of lysozyme, alkaline phosphatase and protease was confirmed for the first time in the mucus of the Senegalese sole. The activity of lysozyme ranged from 0.05 to 0.08 U/mg protein at the 4 weeks sampling and 0.05 to 0.06 U/mg protein at the end of the trial. Lysozyme activity was not significantly affected (P>0.05) by the various dietary treatments or by the duration of feeding (Figure 9).

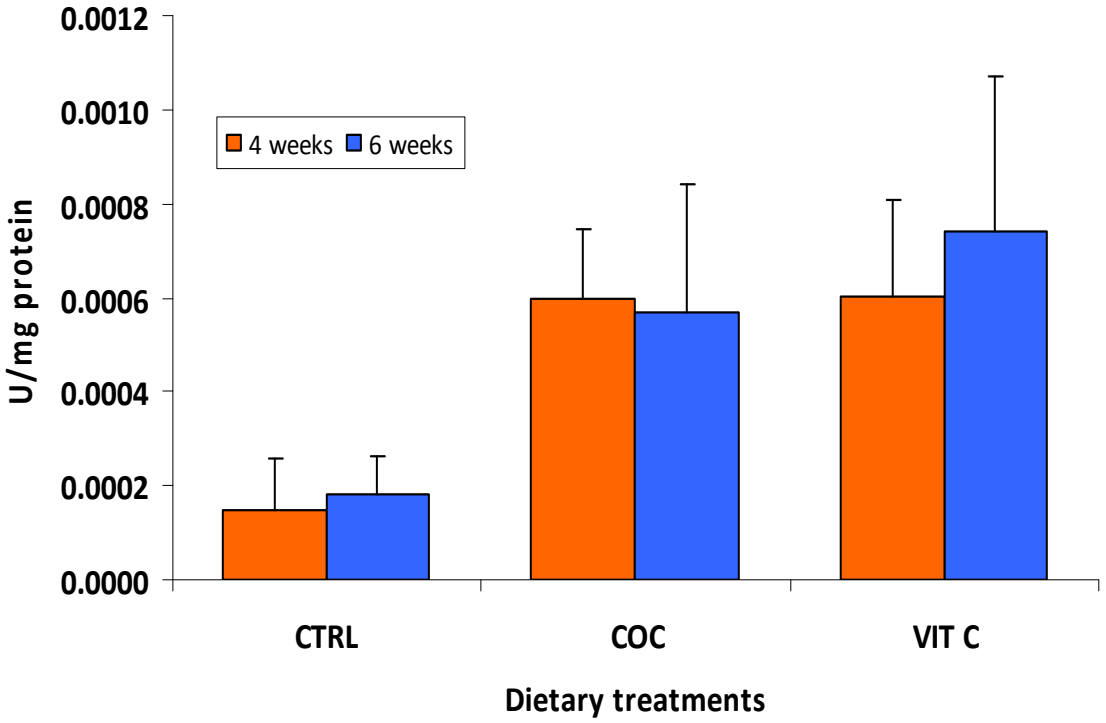


ANOVA Two-factors	Statistical analysis		
95% confidence level	Diet	Time	Interaction
Lysozyme	0.211	0.050	0.059

Figure 9. Lysozyme activity in the mucus of sole fed the various dietary treatments during 4 and 6 weeks. Bars are presented as mean ± standard deviation (n=6). Values refer to probability levels from ANOVA Two-factor statistical analysis.

Despite the extremely low levels of alkaline phosphatase (AP) found in the mucus of Senegalese sole, results show that AP activity in fish fed coconut oil and vitamin C rich diets was significantly higher (P<0.05) than that found in fish fed the control diet (Figure 10). After 4 weeks of experimental feeding, alkaline phosphatase levels for the CTRL treatment (0.001 U/mg protein)

were six-fold increased in both COC and VIT C treatments. At the end of the trial (6 weeks of experimental feeding), this situation was maintained, but such increase on AP activity seen in fish fed diets COC and VIT C was only three-fold in relation to CTRL diet. The duration of feeding (either 4 or 6 weeks) had no significant effect (P>0.05) on alkaline phosphatase activity. Similarly, the interaction between the two variables (dietary treatment and duration of feeding) did not prove statistical significant at a 95% confidence level.

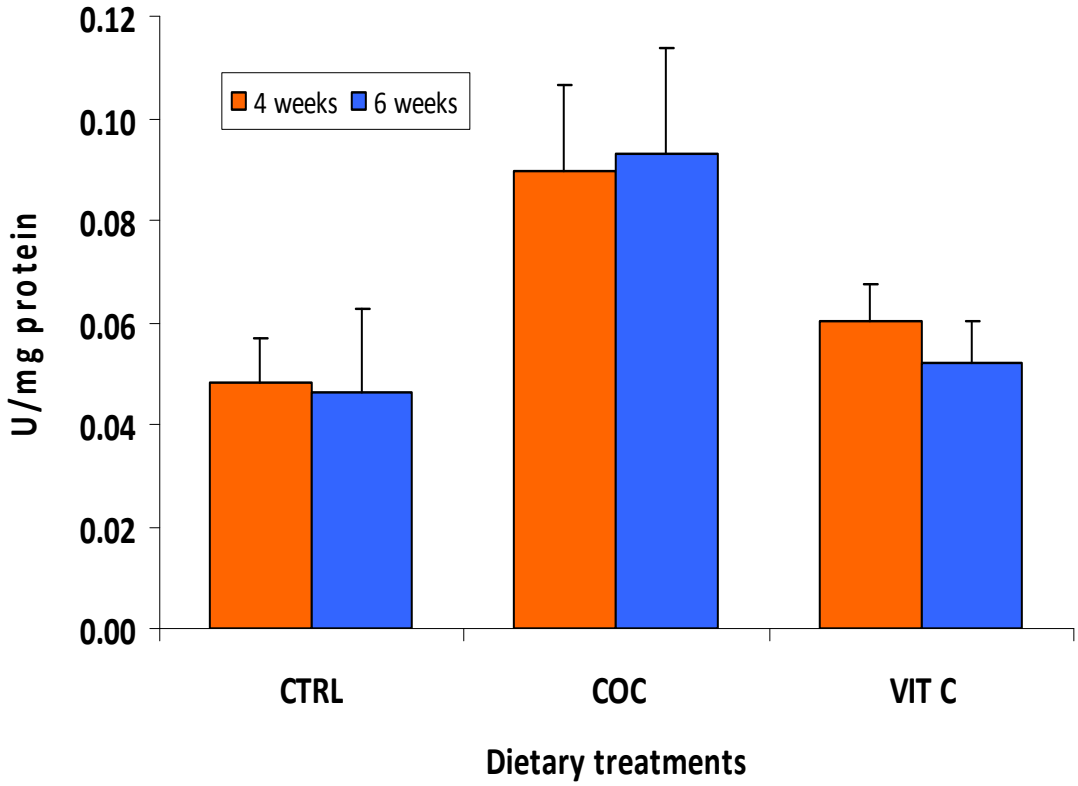


ANOVA Two-factors	Statistical analysis		
95% confidence level	Diet	Time	Interaction
Alkaline phosphatase	***0.000***	0.507	0.685
Means comparison by Student-Newman-Keuls test			
Diet	a	b	b

Figure 10. Alkaline phosphatase activity in the mucus of sole fed the various dietary treatments during 4 and 6 weeks. Bars are presented as mean ± standard deviation (n=6). Values refer to probability levels from ANOVA Two-factor statistical analysis.

At both 4 and 6 weeks of experimental feeding, the proteolytic activity in the mucus of Senegalese sole fed the CTRL diet was 0.05 U/mg protein (Figure 11). The activity of proteases in the mucus of

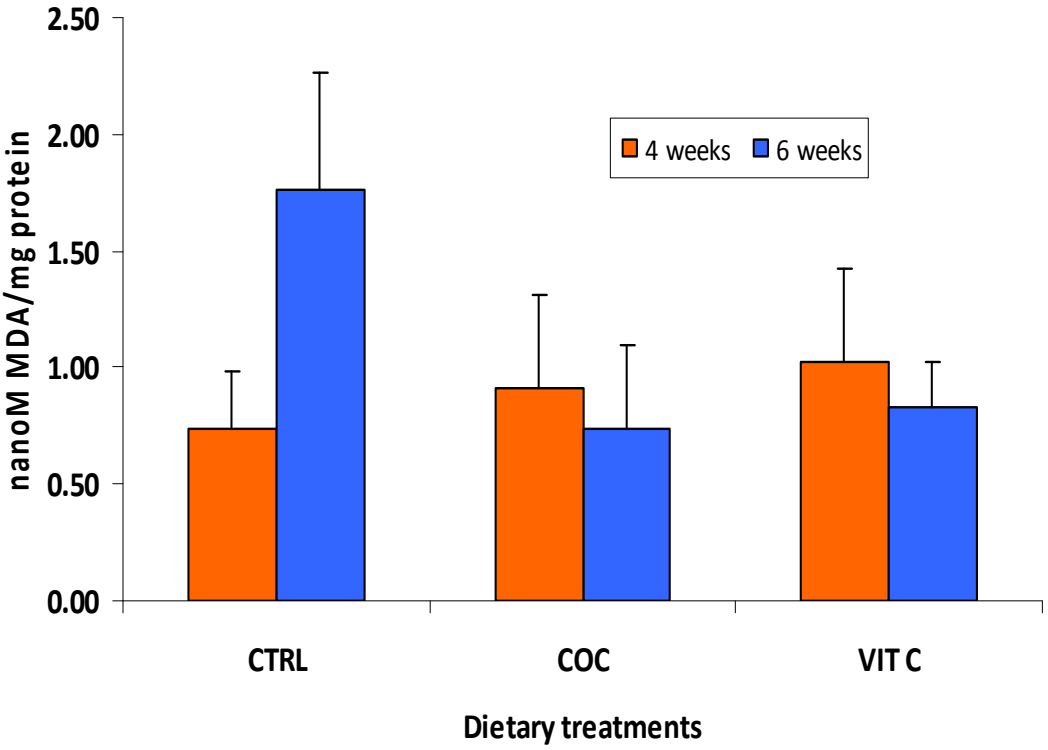
fish fed the VIT C supplemented diet ranged from 0.05 to 0.06 U/mg protein and were not significantly affected by either dietary treatment or duration of feeding. However, sole fed the diet incorporating coconut oil (COC) showed a significant increase ($P<0.05$) of proteolytic activity in their mucus. Both the duration of feeding (either 4 or 6 weeks) and its interaction with the dietary treatment had no significant effect ($P>0.05$) on mucus proteolytic activity.



ANOVA Two-factors	Statistical analysis		
95% confidence level	Diet	Time	Interaction
Protease activity	***0.000***	0.653	0.645
Means comparison by Student-Newman-Keuls test			
Diet	a	b	a

Figure 11. Proteolytic activity in the mucus of sole fed the various dietary treatments during 4 and 6 weeks. Bars are presented as mean ± standard deviation (n=6). Values refer to probability levels from ANOVA Two-factor statistical analysis.

Data regarding the lipid oxidation potential (measured as TBARS) in mucus of Senegalese sole fed the various experimental diets during 4 and 6 weeks are presented in Figure 12. After 4 weeks of feeding, TBARS levels varied between 0.74 and 1.03 nanomoles MDA/mg protein, with the lowest values being found in fish fed the CTRL diet. However, after 6 weeks of feeding and if comparing to the CTRL treatment, feeding sole with either coconut oil or a high dose of vitamin C significantly reduced ($P<0.05$) mucus susceptibility to lipid oxidation. Despite the fact that the duration of experimental feeding has not affected significantly TBARS, its interaction with the dietary treatments was highly significant statistically.



ANOVA Two-factors	Statistical analysis		
95% confidence level	Diet	Time	Interaction
Lipid oxidation (TBARS)	***0.026***	0.093	***0.000***
Means comparison by Student-Newman-Keuls test			
Diet	b	a	a

Figure 12. Lipid oxidation (TBARS) in the mucus of sole fed the various dietary treatments during 4 and 6 weeks. Bars are presented as mean ± standard deviation (n=6). Values refer to probability levels from ANOVA Two-factor statistical analysis.

3.3. Antibacterial activity

The growth inhibitory effects of experimental mucus extracts against selected pathogens commonly occurring in a marine environment are presented in Table IV. The epidermal mucus extracts of Senegalese sole showed antibacterial activity against a series of known marine pathogen bacteria. A moderate inhibitory effect was observed against *Aeromonas hydrophila* by mucus of fish fed the CTRL and the VIT C treatments. However, the mucus from fish fed the coconut oil diet (COC) showed a potent antibacterial effect against *Aeromonas hydrophila*. For both *Aeromonas salmonicida* and *Listonella anguillarum*, mucus from all dietary treatments showed an important growth inhibitory effect, but again highest inhibitory potential was observed in the mucus of fish fed the coconut oil diet. *Vibrio alginolyticus* was not inhibited by the mucus of the CTRL treatment, moderately by the VIT C treatment and strongly by the COC treatment. The mucus from fish fed coconut oil was the only extract that showed and inhibition effect on *Yersinia ruckeri*. Overall, results show that mucus derived from fish fed the coconut oil based diet showed the highest antibacterial effect on the selected marine pathogens.

Table IV. Inhibition of bacterial growth by 100 µl of epidermal mucus of sole.

	CTRL	COC	VIT C	Chloramphenicol	Oxytetracycline
Aeromonas hydrophila	+	+++	+	+++	+++
Aeromonas salmonicida	++	+++	++	+++	+++
Listonella anguillarum	++	+++	++	+++	+++
Vibrio alginolyticus	-	++	+	+++	-
Yersinia ruckeri	-	++	-	+++	+++

(-): no activity; (+): $0 < D < 2$ mm; (++): $2 \leq D < 7$ mm; (+++): $D \geq 7$ mm.

D: Diameter of the inhibition zone in millimeters not including the diameter of the disc.

4. Discussion

The aquatic environment is a complex ecosystem, which obscures the distinction between health, sub-optimal performance and disease. During disease outbreaks, the underlying cause is often difficult to ascertain and is usually the end result of a series of linked events involving environmental factors, health condition of the stocks, presence of an infectious agent and/or poor husbandry and management practices. The whole aquatic production environment, including ecological processes, must be taken into consideration. Therefore, an aquatic system health management approach needs to be developed to replace the more traditional pathogen-focused approach applied traditionally to disease diagnosis.

Fish are in constant interaction with their aquatic environment, which contains a large range of non-pathogenic and pathogenic microorganisms. The epidermal mucus secretions and epidermis act as the first biological barrier between fish and the potential pathogens present in the environment (Shephard, 1994). Several studies suggest the protective role of the mucus and its components in various fish species (Aranishi, 1999; Palaksha et al., 2008). The presence in the mucus of certain hydrolytic enzymes including lysozyme, alkaline phosphatase and proteases has been studied in a number of fish species (Ross et al., 2000; Fast et al., 2002; Subramanian et al., 2007), but to date no published information was available on the presence of such factors in the mucus of Senegalese sole.

Presently, the main factor hampering the successful implementation of large scale cultivation of sole it is the high sensitivity of the post-larvae and juveniles to stressful situations commonly found in aquaculture activities, such as grading, weighing or initial feeding with artificial diets. Such stressful conditions are often associated to mass mortalities due to a high susceptibility to opportunistic pathogens, mainly *Flexibacter*. As a benthonic species, sole lives in direct contact with the bottom substrate. It has been suggested that by this fact, benthonic species, such as hagfish, may present higher levels of innate immune factors in skin mucus (Subramanian et al., 2007). It was therefore our objective to characterize the epidermal mucus of sole in terms of its

content of selected immune-associated factors. Furthermore, this study also evaluated the role of certain nutrients (vitamin C and coconut oil as a source of lauric and capric fatty acids) as modulators of the activity of some mucus immune factors.

In the present study, mucus samples were collected by placing individual sole in plastic bags and gently shaken the fish with NaCl 100 mM. An alternative methodology, by means of skin scrapping of recently killed fish is described by Mozumder (2005). However, we have adopted the previous methodology given that Subramanian et al. (2007) suggested that such method of mucus collection yielded samples which accurately represented what was naturally present in the fish skin mucus at the time of sampling.

Growth performance

Specific growth rate values ranged from 0.38 to 0.63 %/day and neither weight gain nor growth rates were significantly affected ($P>0.05$) by vitamin C supplementation or coconut oil inclusion. Li et al. (2007) found also no relationship between vitamin C supplemented diets (33 to 424 mg/kg feed) and the growth rates of juvenile Atlantic salmon. This work is in accordance with our results in the vitamin C treatment. Similarly to our results with sole, no effect on weight gain or growth was observed in climbing perch (*Anabas testudineus*) fed diets containing 20% coconut oil. However, given the relatively short duration of the experimental period, it was not a primary objective of this study to induce a growth enhancement in fish. Furthermore, the usually high weight dispersion found in sole rearing makes it hard to infer statistical significance from our data.

Immune factors

As a component of the innate immune mechanism, the epidermal mucus plays a dual role in fish. First, by being continuously produced and sloughed off it prevents pathogen adherence (Pickering, 1974). Secondly, it also serves as a repository of numerous innate immune factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, proteolytic enzymes

and various other antibacterial proteins and peptides (Shepherd, 1994; Cole et al., 1997; Ingram, 1980).

Lysozyme is an enzyme which acts as a hydrolase by breaking down the beta-(1-4) linkages in the peptidoglycan layer of bacterial cell walls. Bacteria are either destroyed directly or opsonized, enabling its destruction through phagocytosis. While Gram-positive bacteria are more sensitive to lysozyme than Gram-negative bacteria in mammals, fish lysozyme is capable of responding to both Gram-positive and Gram-negative bacteria. Lysozyme often works simultaneously with other mechanisms of the non-specific immune system, such as complement and macrophage activity (Subramanian et al., 2007).

Lysozyme is continuously expressed by neutrophils, monocytes and macrophages, although lysozyme activity has been found to increase with a stimulation of the immune system. Changes in lysozyme activity are common once a fish becomes infected with a pathogen, depending on the level of infection. Lysozyme has been reported in several fish tissues such as serum, plasma, lymph, spleen, liver, skin, mucus, gills, muscle, ovary and eggs and other organs or tissues (Fänge et al., 1976; Takemura & Takano, 1995). The enzyme may act as a first encounter defense to bacteria by existing externally on the fish, such as in the skin mucus and gills. These defense strategies, along with the internal function of lysozyme within the major organs, contribute greatly to preventing bacteria from causing infection before it starts.

In this work, the activity of lysozyme in epidermal mucus of Senegalese sole ranged between 0.05 and 0.08 U/mg protein. This range of activities is considerably lower than the values reported previously in other marine species. Several authors have quantified lysosyme activity in fish mucus. Subramanian et al. (2007) studied several fish species and observed different values of lysozyme activity, specifically 63.1 U/mg protein in Atlantic cod, 88.1 U/mg protein in haddock and 124.7 U/mg protein in hagfish. Fast et al. (2002) reported a lysosyme activity value of 19.1 U/mg protein in coho salmon, 65.0 U/mg protein in rainbow trout and 13.6 U/mg protein in Atlantic salmon. This high variation in lysozyme activity could be related to several factors such as responses to handling

stress, sexual maturity, dietary status, sex and genotype (Balfry & Iwama, 2004). Furthermore, large variations in lysosyme activity have been associated with species specific evolutionary adaptations to different environmental conditions (Subramanian et al. (2007).

Given the low levels of lysozyme activity found in sole epidermal mucus, during this study, a great effort has been deployed to validate the quantification assay of lysozyme. Aspects such as the interference of ionic salts in the assay have been evaluated. However, several previous works on the lysozyme assay in the mucus of both freshwater and marine fish species refer that a sample desalting step is not required for better accuracy (Subramanian et al., 2007).

In the current study, lysozyme activity was not significantly affected ($P>0.05$) by the various dietary treatments or by the duration of experimental feeding. The influence of dietary ascorbic acid (vitamin C) on the immune response has been related to its antioxidant activity as a free radical scavenger (Kumari & Sahoo 2005). Similarly to our findings, lysozyme activity remained unchanged in Asian catfish (*Clarias batrachus*) fed various vitamin C levels (0, 500, 1000 and 2000 mg AA equivalent/kg diet) during 2, 4, 6 and 8 weeks (Kumari & Sahoo, 2005). However, there are numerous studies in a wide range of species, demonstrating that lysozyme levels can be altered by feeding elevated doses of vitamin C (Montero et al., 1999; Ai et al., 2006).

To our knowledge the role of coconut oil or more precisely of its main constituent, the lauric acid, on the immune response of fish has never been evaluated. Generally, there are three mechanisms by which dietary fatty acids affect disease resistance and immune response. The first is through their influence on the lipid composition of cell membranes. The second mechanism by which dietary fatty acids may affect the immune system involves alteration of signal transduction, possibly due to the effects on protein kinase C. Finally, the third mechanism is the production of immunologically active eicosanoids from non-esterified arachidonic acid, eicosapentaenoic acid (EPA), decosahexaenoic acid (DHA), and possibly other polyunsaturated fatty acid precursors (Bell et al., 2004). The lipid composition of juvenile turbot was modified by changes in dietary lipid sources (Balbry & Higgs, 2001), but no differences were found in lysosyme activity in this study.

Even though the experimental treatments tested in this work did not have a significant effect in lysozyme activity, it appears that the duration of feeding and the interaction between this variable and the dietary treatment were very close to statistic significance (0,050 and 0,059 respectively). It is possible that initially (at 4 weeks) both treatments induced an increase in lysosyme activity, but with time the effect diminished and the activity returned to normal levels. According to Kumari & Sahoo (2005), the non-specific immune parameters as well as percent survival were enhanced as a result of high dietary vitamin C level, particularly at 500 mg/kg diet, although this initial increase was not maintained but returned to the control levels after 4 weeks of feeding.

The precise function of alkaline phosphatase (AP) in the innate immune mechanism is yet to be elucidated. Iger & Abraham (1997) demonstrated its production by the epidermal rodlet cells of the common carp and rainbow trout in response to various stress factors. A protective role for AP specific activity was suggested by Iger & Abraham (1990) when AP was observed in the epidermal mucus cells during wound healing in an experimentally wounded common carp. Elevated AP levels were observed in the mucus of Atlantic salmon infected with the ectoparasite *Lepeophtheirus salmonis* (Ross et al., 2000).

In the present work, the alkaline phosphatase activity in sole mucus ranged from 0.0001 to 0.0007 U/mg protein. This enzyme has already been quantified in several species of fish, such as haddock, Atlantic cod and hagfish (respectively 0.9, 0.32 and 1.32 U/mg protein by Subramanian et al., 2007). Fast et al. (2002) found alkaline phosphatase activities of 1.04, 0.75 and 0.45 U/mg protein in rainbow trout, coho salmon and Atlantic salmon, respectively. These values are considerably higher than the ones found here for Senegalese sole. Palanksha et al. (2008) reported that alkaline phosphatase can act individually or in cooperation with other immune substances in the mucus in defending against pathogens or in healing wounds on the body surface. The low activities found here (possibly also with lysozyme) seems to suggest that, these enzymes may have been redirected to other pathways of the immune system.

Both dietary treatments used in this study significantly increased alkaline phosphatase activity. After 4 weeks, vitamin C supplementation and coconut oil rich diet induced a six-fold increase of alkaline phosphatase activity in the epidermal mucus of sole. Information about the modulation of alkaline phosphatase activity by dietary vitamin C levels or dietary lipid sources is extremely scarce in fish. Serum alkaline phosphatase activity was found to be lowered in Channel catfish fed vitamin C-free diets (Wilson & Poe, 1973). Previous studies with rodents showed also that serum and bone alkaline phosphatase activity was decreased during vitamin C deficiency (Mahmoodian et al., 1996). Furthermore, this enzyme seems to be strongly influenced by the type of lipids used in the diet. Alkaline phosphatase is an enzyme known to be intimately associated with the hydrophobic core of membranes. Thus, alkaline phosphatase is often used as a marker of membrane functionality. For instance, the alkaline phosphatase activity was found to be increased in coconut oil-fed rats when compared to fish oil-fed rats (Wahnon et al., 1992)**.** The duration of feeding as a variable was also very close to statistical significance. Knowing that several weeks of feeding are required to alter the fatty acid composition of cellular membranes in fish, it would be interesting to assess the alkaline phosphatase activity over a longer feeding period.

Proteases can act directly on pathogens or prevent invasion indirectly by modifying mucus consistency, resulting in an increased sloughing of mucus and pathogen removal from the body surface, and activating and enhancing the production of various immunological components (Aranishi et al., 1999). In Senegalese sole, proteolytic activity in the mucus varied between 0.048 and 0.093 U/mg protein. Subramanian et al. (2007) found highly variable values of protease activity, specifically 15.1 U/mg protein in Atlantic cod, 10.8 U/mg protein in haddock and 818.0 U/mg protein in hagfish. Fast et al. (2002) reported protease activity of 0.036 U/mg protein in coho salmon, 0.051 U/mg protein in rainbow trout and 0.026 U/mg protein in Atlantic salmon. The results of the last author are of the same magnitude of those obtained here with sole. Whether this wide variability on the proteolytic capacity of mucus in the different species is a result of differences in the evolutionary or genetic adaptations of these species it is not clear. Furthermore, the biochemical substances in mucus have been showed to differ depending on ecological and

physiological conditions, such as salinity, pH, handling stress and developmental stage (Subramanian et al., 2008).

This study observes an increased protease activity in the fish fed with the diet supplemented with coconut oil but not in those fed with the vitamin C supplemented diet. In humans, high doses of vitamin C are often associated to a reduction of specific protease activities, by enhancing the effect of possibly present inhibitors (Fukal et al., 1986). The mechanism by which coconut oil would induce an increased protease activity remains to be elucidated.

Reactive oxygen species (ROS) are able to start the lipid peroxidation process which potentially dangerous in fish, since they contain a high percentage of n-3 polyunsaturated fatty acids, in particular EPA and DHA, which account for most of seafood's nutritional quality, and for the health of farmed fish (Passi et al., 2004). Mucus TBARS levels in sole varied between 0.74 and 1.76 nM MDA/mg of protein. It has to be pointed out that such parameter has never been assessed in fish mucus.

In this study, there was a significant decrease in TBARS in the mucus of fish fed diets supplemented with coconut oil, as well as those fed with vitamin C supplements. Both supplements have proved to reduce the presence of oxidized lipids in Senegalese sole mucus. Antioxidant defenses in fish are strongly dependent on nutritional factors. Dietary levels of lipids and some vitamins have been reported to influence antioxidant defenses and oxidative status of fish (Martínez-Álvarez et al., 2005). Kumari & Sahoo (2005) reported that the influence of ascorbic acid on the immune response may be related to its antioxidant activity as a free radical scavenger, protecting cells from auto-oxidation and maintaining their integrity for an optimal functioning of the immune system. Vitamin C is involved in several enzymatic processes, so it is difficult to determine the exact mechanism of action behind the stimulation of the immune response. Our results are in accordance with this author, and we can conclude that this antioxidant action extends to fish mucus. Coconut oil as also proved to have antioxidant action in this study. Mourente et al. (2002) showed that diets containing oxidized oil significantly affected the activities of liver antioxidant

defence enzymes of gilthead sea bream. In Senegalese sole, Rueda-Jasso et al. (2004) reported that activity levels of antioxidant enzymes CAT and SOD were higher in livers of fish fed diets with a high lipid level. Despite not measured, it can be expected that the replacement of fish oil by coconut oil would reduce the overall content of n-3 PUFA in sole tissues and therefore its susceptibility to oxidation processes. Whether this assumption is true in epidermal mucus remains to be confirmed.

Antibacterial activity

Epidermal mucus of fish has been shown to play a significant role in host defense against bacteria and viruses. Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermal mucus in a wide variety of species were also studied and confirmed by Hellio et al. (2002).

The epidermal mucus extracts of Senegalese sole showed antibacterial activity against a series of known marine pathogen bacteria. In this work, the mucus of fish fed the coconut oil diet showed a strong antibacterial effect against *Aeromonas hydrophila, Aeromonas salmonicida* and *Listonella anguilarum*, and a moderate growth inhibitory effect against *Yersinia ruckeri* and *Vibrio anginolyticus*. Sole fed the control and the vitamin C supplemented diet showed a moderate inhibitory effect against *Aeromonas salmonicida* and *Listonella anguilarum*. Antibacterial activity present in the skin mucus of turbot, gilthead seabream and European seabass against *Pasteurella piscicida* and *Flexibacter maritimus* has been assayed (Magariños et al., 1995). Using assays on agar plates, none of the mucus samples from the above species showed antibacterial activity against *F. maritimus* isolates. Turbot mucus inhibited the growth of *P. piscicida*, but mucus of seabream and seabass did not. The bactericidal properties of the mucus were lost after a heat treatment at pH 3.5 and skin mucus samples displayed activity against *Staphylococcus aureus* ATTC 25923, a strain resistant to lysozyme. These findings indicate that thermolabile substances other than lysozyme are responsible for the antibacterial activity in mucus of marine fish. Austin and McIntosh (1998) demonstrated that the antimicrobial compounds present in the mucus layer of rainbow trout predominantly inhibited the growth of *Aeromonas hydrophila*.

However, knowledge regarding the underlying mechanisms involved in such defenses is limited. Indeed, Ebran et al. (1999) identified a strong antibacterial activity well correlated with pore-forming properties against several bacterial strains and suggested that fish secreted antibacterial proteins able to permeabilize the membrane of the target cell and thus act as a defence barrier. Bergsson et al. (2005) observed that components of Atlantic cod mucus were active against both Gram-positive and Gram-negative bacteria. These authors identified four evolutionarily conserved cationic, bactericidal polypeptides (histone H2B and three 60S ribosomal proteins, L40, L36A and L35) and numerous unidentified antimicrobial components from the skin mucus of Atlantic cod. A 33-amino acid pore forming peptide pardaxin was one of the early discovered antimicrobial peptides. Lazarovici et al. (1986) isolated pardaxin from the secretions of the Red Sea Moses sole fish, *Pardachirus marmoratus*. It has been reported that this peptide has a helix-hinge-helix structure similar to cecropin and mellitin, and it is an excitatory toxin that possesses high antibacterial activity. A 25-amino acid residue linear antimicrobial peptide, pleurocidin, was found in the skin mucous secretions of the winter flounder *Pleuronectes americanus* and other flatfish species (Cole et al., 1997; Patrzykat et al., 2003). Three antimicrobial proteins were isolated from catfish skin in 1998. The molecular masses of the proteins were 15.5, 15.5, and 30 kDa. Amino acid composition and amino acid sequence data suggested that these proteins are closely related to histone H2B proteins. These H2B-like proteins inhibited the growth of *A. hydrophila* and *Saprolegnia* spp (Robinette et al., 1998).

Some mucus enzymes may also influence the innate defense by activating the expression of genes that encode proteins such as antimicrobial peptides and complement proteins, and could thereby impart antimicrobial activity through indirect mechanisms. For example, cathepsin D has been shown to be involved in the production of the antimicrobial peptide, parasin I, in the mucus of catfish and Japanese flounder (Aranishi & Mano, 2000; Cho et al., 2002). Pleurocidin, an antimicrobial peptide isolated from flounder skin mucus was found to act synergistically with lysozyme to enhance disease resistance in coho salmon (Patrzykat et al., 2003).

Knowledge about the effects of dietary nutrients on the modulation of the antibacterial potential in fish mucus is extremely scarce, not to say inexistent. However, studies with higher vertebrates and humans clearly show that lauric acid, a main constituent of coconut oil, has strong antibacterial activity against a wide range of pathogenic bacteria (Rouse et al., 2005; Carpo et al., 2007). Besides lauric acid and its derivatives, other fatty acids moieties such as sapienic acid and the sphingoid bases are all present at the skin surface, and all have documented antibacterial activity against various potential skin pathogens (Drake et al., 2008). Little is known about the effect of a vitamin C dietary supplementation on epidermal antibacterial defences. A positive relationship between the wound healing response and ascorbate level in the skin has been reported in rainbow trout (Wahli et al., 2003), gilthead seabream (Alexis et al., 1997) and channel catfish (Lim & Lovell, 1978). Rainbow trout showed a significant decrease in tissue ascorbic acid concentration following wounding, suggesting a depletion of the tissue vitamin pool as the healing process begins (Wahli et al., 2003). These same authors suggested that vitamin C is used not only for collagen biosynthesis, but also for the regulation of physiological conditions in the healing process, such as re-vascularisation, dermal structure and fibrous proliferation.

5. Conclusions

The epidermal mucus secretions of Senegalese sole serves as repository of some innate immune factors such as lysozyme, alkaline phosphatase and proteolytic enzymes, and possesses antibacterial activity against several marine pathogen bacteria.

The modulation of selected mucus immune parameters and its antibacterial activity through dietary factors seems possible in Senegalese sole. A four weeks period of feeding seems enough to induce such changes.

Selected mucus non-specific immune parameters were significantly affected by dietary treatments:

- Mucus proteolytic activity was significantly enhanced in fish fed diets rich in coconut oil.
- Activity of alkaline phosphatase in the mucus of sole fed coconut oil and vitamin C rich diets was significantly higher than that found in fish fed the control diet.
- In comparison to the control diet, feeding sole with coconut oil and vitamin C significantly reduced mucus susceptibility to lipid oxidation (TBARS).
- Epidermal mucus of sole showed antibacterial activity against a series of marine pathogen bacteria. Highest inhibitory action was associated to mucus extracts derived form coconut oil fed fish.

Given the beneficial effects observed with the usage of coconut oil as a dietary lipid source, it would be interesting to validate such results in a more precise way. Namely, test the effect of graded levels of purified lauric acid in the diet. Further research is needed to determine the processes associated to the nutritional modulation of immune response in sole and evaluate to what extent the observed beneficial changes contribute to an enhanced disease resistance of sole. It would be important to test more parameters of the innate immune system, in order to broaden the knowledge on the role of this important mucus barrier in Senegalese sole.

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